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Lil3 assembles as chlorophyll-binding protein complex during deetiolation

Veronika Reisinger, Matthias Plöscher, Lutz A. Eichacker*

Ludwig-Maximilians-University Munich, Department Biology I, Menzingerstrasse 67, 80638 Munich, Germany

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Abstract Dark-grown angiosperm seedlings are etiolated and devoid of chlorophyll. Deetiolation is triggered by light leading to chlorophyll dependent accumulation of the photosynthetic machinery. The transfer of chlorophyll to the chlorophyll-binding proteins is still unclear. We demonstrate here that upon illumination of dark-grown barley seedlings, two new pigment-binding protein complexes are de novo accumulated. Pigments bound to both complexes are identified as chlorophyll *a* and protochlorophyll *a*. By auto-fluorescence tracking and mass spectrometry, we show that exclusively Lil3 is the pigment-binding complex subunit in both complexes.

Keywords: Etioplast; Chlorophyll synthesis; Chlorophyll protein complexes; Assembly; Lil 3

1. Introduction

The pigment chlorophyll *a* (Chl) is a prerequisite for the accumulation of Chl-binding photosystem proteins [1,2]. Chl-binding proteins constitute an integral part of both photosystem I (PS I) and photosystem II (PS II) and represent essential elements in the photosynthetic electron transfer chain. To study the biogenesis of photosystem protein complexes, deetiolation of dark-grown angiosperms has been developed as an important tool for developmental studies, especially in graminеae. In darkness, primary leaf cells of grasses like maize, rice or barley, develop etioplasts instead of chloroplasts. Etioplasts are characterized by the absence of Chl but accumulation of a large internal membrane phase harbouring the light-dependent enzyme NADPH/H^+-protochlorophyllide oxidoreductase (POR) and the pigment protochlorophyllide *a* (Pchlide) [3]. In etioplasts, POR assembles in a ternary complex composed of NADPH and the non-esterified Chl precursor Pchlide [4]. The induction of Chl synthesis starts with the conversion of Pchlide to chlorophyllide *a* (Chlide) by POR. Here, catalysis requires light as substrate, whereas for the subsequent esterification of Chlide with phytylpyrophospate to Chl by Chl synthase (CHS), catalysis is light independent. Interestingly, plastid encoded Chl-binding proteins are translated at a high rate in the absence of Chl; however, accumulation of the photosystem complexes is strictly regulated by Chl [5]. Still, the D2 protein and the cytochrome (Cyt) b_6f protein complex have been found to be an exception to this rule. The D2 protein is expressed in darkness and accumulates to a moderate level in a low molecular mass assembly intermediate despite the absence of Chl [6], whereas the dimeric Cyt b_6f complex has been shown to assemble into its mature form by binding the Chl-derivative protochlorophyll *a* (Pchl) in etioplasts [7]. Also, other proteins of the photosynthetic machinery like the ATP synthase (ATPase) have been detected in their mature state in etioplasts [7].

Upon illumination of dark-grown seedlings, etioplasts differentiate into photosynthetically active chloroplasts within a few hours. After 4 h of illumination, the onset of linear electron transfer has been described and it was shown that induction of PS I activity precedes activity of PS II [8]. However, the chronology for assembly of the Chl-binding proteins still remains an enigma. Here, we show that light-induced synthesis of Chl triggers assembly of the light-harvesting like protein Lil3 in vivo.

2. Materials and methods

2.1. Membrane protein complex isolation

Barley seedlings were grown in a light-proof growth chamber for 4–5 days. In case of illumination, seedlings were illuminated for 10 s directly before plastid isolation. In brief, plastid isolation and membrane complex isolation was carried out on ice as described previously [7]. After plastid isolation, plastids were lysed and membranes were washed twice in TMK buffer (10 mM Tris–HCl, pH 8.5; 10 mM $MgCl_2$; 20 mM KCl). Membrane protein complexes were solubilized in 0.38% (w/v) *n*-dodecyl-β-D-maltoside, 0.64% (w/v) digitonin and 0.006% (w/v) lithium dodecyl sulfate.

2.2. 2D LN/SDS–PAGE and protein visualisation

Solubilized membrane protein complexes were separated by LN/SDS–PAGE [7]. After electrophoresis, pigment-binding proteins were visualised by fluorescence scanning in a Typhoon Trio (excitation 633 nm/670 BP30 emission filter). After auto-fluorescent detection, the LN/SDS–PAGE was stained with Coomassie colloidal and the spots corresponding to the auto-fluorescent spots were cut for protein identification.

2.3. Pigment analysis

For absorption spectroscopy, auto-fluorescent bands were cut after LN-PAGE and an absorption spectrum was recorded from 400 to 700 nm from four combined bands.

Pigments were eluted from the auto-fluorescent bands in dimethylformamide at 4 °C for 1 h. The pigment containing solution was extracted from the gel by centrifugation and concentrated in a vacuum concentrator. Reference pigments and extracted pigments were dissolved in the mobile phase solution (acetone:methanol:H_2O

*Corresponding author. Fax: +49 0 89 17861 209.
E-mail address: lutz.eichacker@lrz.uni-muenchen.de (L.A. Eichacker).

Abbreviations: Lil, light-harvesting like; PS, photosystem; Chl, chlorophyll; Pchl, protochlorophyll; Pchlide, protochlorophyllide; Chlide, chlorophyllide; LN-PAGE, LDS-native polyacrylamide gel electrophoresis

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(20:30:1)) and spotted on a HPTLC RP-8F_{254} plate. After thin layer chromatography (TLC), the TLC plate was read out in the Typhoon Trio (excitation 633 nm/670 BP30 emission filter).

2.4. Protein identification

After tryptic digestion [9] of proteins separated by 2D LN/SDS–PAGE and detected by auto-fluorescence, peptides were analysed by ESI-MS/MS-fragmentation and de novo sequencing of the peptide fragment spectra. All peptide sequences were used for database search using the frame "fasta3" from the European Bioinformatics Institute.

3. Results

Accumulation of the first Chl-binding protein complex was investigated after illumination of etiolated angiosperm seedlings. For high sensitivity of complex detection and resolution, the fluorescent properties of protein complexes were scanned after electrophoretic separation. Therefore, etiolated seedlings were either illuminated (L) or maintained in darkness (D). Etioplasts were isolated, and membranes were prepared after lysis of the etioplasts before membrane protein complexes were solubilized and separated by colourless native electrophoresis (LN-PAGE). Afterwards, the Chl-binding protein complexes were detected by laser excitation at 633 nm in the Typhoon Trio and the auto-fluorescent band pattern from both developmental states were compared (Fig. 1, D vs. L). In the etioplast, two auto-fluorescent bands were detected (Fig. 1D). We recently demonstrated that the auto-fluorescence of both complexes originates from the association of Pchl *a* with two different assembly stages of the Cyt b_6f complex [7]. Here, molecular mass and subunit determination of the protein complexes at about 140 kDa and 270 kDa indicated a monomeric and dimeric assembly state (Fig. 1D, Cyt b_6f (1), Cyt b_6f (2)). Upon illumination of the etiolated seedlings, two additional auto-fluorescent protein complexes were identified (Fig. 1L) at a molecular mass of 210–250 kDa and 160–180 kDa which were labelled as complex **1** (1) and complex **2** (2), respectively (Fig. 1L, **1/2**).

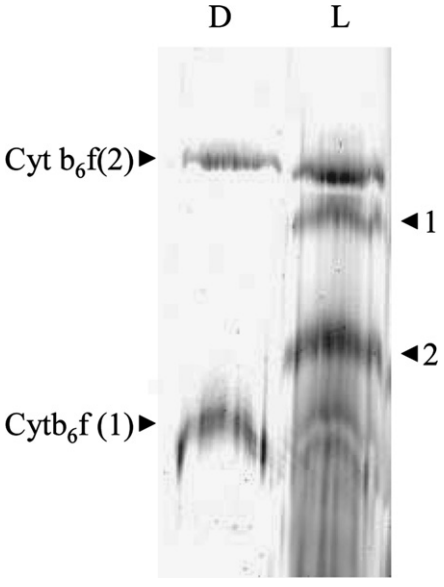

Fig. 1. Comparison of auto-fluorescent complexes in dark-grown and illuminated seedlings. Membrane protein complexes from 2×10^8 etioplasts (D) or plastids isolated from short time illuminated dark-grown seedlings (L) were separated by LN-PAGE and visualised by fluorescence scanning. The dimeric and monomeric Cyt b_6f complex and the de novo accumulated protein complexes (**1/2**) are labelled.

In order to identify the pigments bound to these two de novo assembled complexes, absorbance-spectroscopy and thin layer chromatography (TLC) were used. Absorbance spectra were directly recorded from the native protein complexes in the visible range from 400 to 700 nm at room temperature (Fig. 2). Both absorbance spectra revealed a Q_y transition maximum at 635 nm and 674/675 nm indicating the presence of Pchl and of Chl or their precursors Pchlide and Chlide, respectively [7,10]. Maxima around 422/423 nm were also assigned to Pchl and Chl (Soret bands). Furthermore, spectral analysis around 482 nm indicated the presence of carotenoids in both complexes. As it is known that etioplasts contain large amounts of Pchlide which is first transformed to Chlide, we used TLC to determine if the esterified or the non-esterified precursor form of both pigments was bound to the complexes. Under the conditions used for TLC separation, non-esterified pigments show an explicitly higher mobility than esterified pigments. Pigments isolated from both complexes (**1/2**) were compared with pigment standards Chlide, the esterified counterpart Chl, and Pchl (Fig. 3). Pigments isolated from both complexes showed similar mobility than the esterified pigments Chl and Pchl. In contrast, the non-esterified Chlide exhibited a much higher mobility and was detected near to the front of the mobile phase. We therefore concluded that both de novo assembled complexes bind carotenoid as well as Chl and Pchl.

In order to identify the nature of the pigment-binding protein subunits, we combined LN-PAGE with SDS–PAGE in the next analytical step. A combination of both separation technologies was performed at sufficiently mild conditions to retain secondary protein structures. Hence, binding of Chl to the partially denatured protein subunits could be analyzed by fluorescence scanning. After LN/SDS–PAGE, two auto-fluorescent protein spots corresponding to the two native protein complexes were separated by LN-PAGE and were detected close to the auto-fluorescent subunits Cyt *f* and Cyt b_6 of the dimeric and monomeric Cyt b_6f complexes. By comparison with molecular mass standard proteins, both auto-fluores-

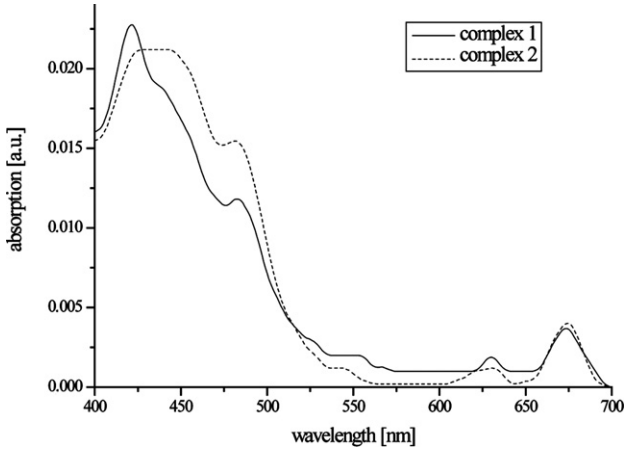

Fig. 2. Absorption spectra of de novo assembled auto-fluorescent complexes. After LN-PAGE, four gel-bands of complex **1** and **2** (Fig. 1, 1 and 2) were cut, combined and an absorption spectrum was recorded.

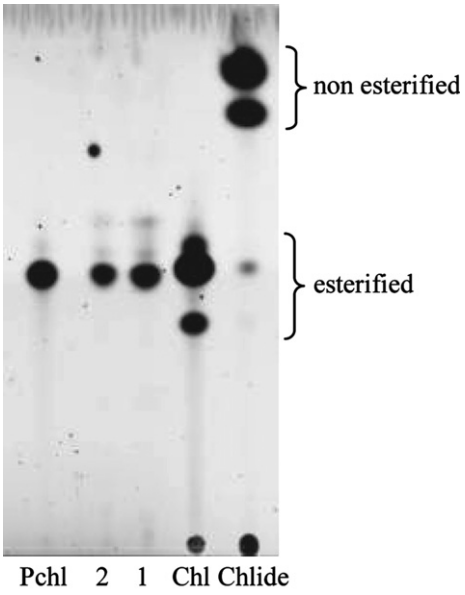

Fig. 3. Characterization of pigments by TLC. Pigments of complex **1** (1) and complex **2** (2) were eluted from the gel, separated by TLC and compared with purified protochlorophyll (Pchl), chlorophyll (Chl) and chlorophyllide (Chlide).

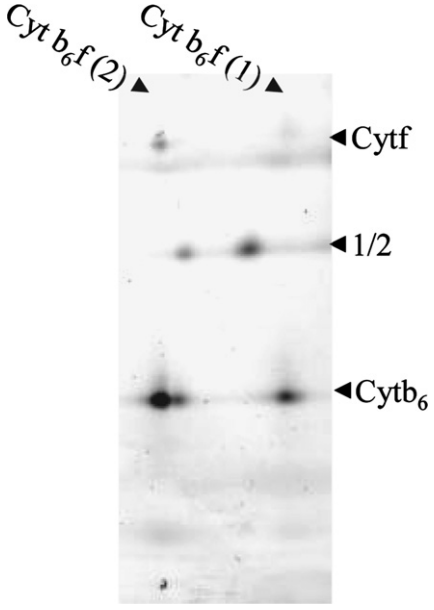

Fig. 4. Auto-fluorescent spot pattern after LN/SDS–PAGE. Membrane protein complexes from 2×10^8 plastids were isolated from short time illuminated dark-grown seedlings and complexes were separated by LN/SDS–PAGE. The gel was scanned for auto-fluorescent spots. Subunits of the Cyt b_6f complex (Cyt *f* and Cyt b_6) and the unknown auto-fluorescent subunit (1/2) of complex **1** and **2** are marked.

cent protein spots were localized at a molecular mass of about 25 kDa (Fig. 4). This indicated that both protein complexes contained the same pigment-binding protein subunit (Figs. 4(1)).

For identification of the protein subunit, the protein spot was prepared for mass spectrometry by tryptic in-gel digestion and peptides were analyzed by ESI-MS/MS. De novo sequencing and FASTS 3 (www.ebi.ac.uk/fasta33/index.html) alignment of the amino acid sequences against the UniProt-Knowledgebase matched a putative Lil3 protein from rice (*Oryza sativa*, Japonica cultivar group, accession number Q6Z2N3) to the four different peptides sequenced (Fig. 4, shaded amino acids). From the genome deduced amino acid sequence of rice, a molecular mass of 27.4 kDa was calculated for the full length Lil3 protein. According to the putative import sequence, determined by the programme ChloroP, Lil3 is a nuclear encoded protein. After subtraction of the putative import sequence which comprehended the first 42 amino acids of the sequence, 22.8 kDa were calculated for the molecular mass of the Lil3 protein (Fig. 4, brackets). Taken together, with at least one bound Chl and/or Pchl molecule, the molecular mass of the auto-fluorescent protein spot is in good agreement with a molecular mass of 25 kDa determined from 2D LN/SDS–PAGE (Fig. 4(1)). Since, we isolated the Lil3 protein with the membrane fraction of the plastids, we scanned the amino acid sequence for the presence of transmembrane helices. Hydrophobicity analysis of the protein by the HMMTOP, TMHMM and the TMPred software identified two transmembrane helices between amino acids 169/170–189 and 194/195–211 (Fig. 5). In addition, the amino acid sequence of the first

>tr|Q6Z2N3|Q6Z2N3_ORYSA Putative Lil3 protein - Oryza sativa (japonica cultivar-group).

[MATSTFSPPASQLSLTRRRLHPGPDLLTLSSPRLRAGLRLAR]AAAGEAPVETVEAPPS
KPEAEPSPAASNGAAVKAEKPPAAAAAPPLPKFRDSRWVNGTWDLR**QFEK**GGAVD
WDAVIDAEARRRKWLEDCPEATSPDEAVVFDTSIIPWWAWMKRFHLPEAEKLNG*RA*
AMIGFFMAYFVDSLT|*GVG*LVDQ*MGNFFCKTLLFVAVAGVL*LVRKNEDIETVKKLIDE**TT**
FYDKQWQATWQDESPSQPKK

485.12 (3+) XXXXXHLPEAEK
615.80 (2+) LIDE**S**TFYDK
617.35 (2+) WVNGTWDLSR
982.45 (2+) **FGNT**GGAVDWDAVIDAEAR

Fig. 5. Analysis of the Lil3 protein. After LN/SDS–PAGE, the Lil3 protein was identified from the auto-fluorescent spots 1 and 2 by de novo sequencing (sequences highlighted in grey). The putative precursor sequence (square brackets), the LHC consensus motif (boxed), and predicted transmembrane regions (italic letters) are marked in the putative protein sequence originating from rice (*Oryza sativa*). The molecular mass and charge state of the peptide sequences identified by mass spectrometry from barley etioplasts are listed. Identified amino acids not conserved in barley and rice are presented in bold letters.

potential transmembrane domain was found to contain a Chl-binding motif characteristic for the Chl *a*/*b*-binding proteins. By further mass spectrometric analysis, no other peptide sequences of any other protein could be identified. We therefore concluded that the auto-fluorescent protein spot in the second dimension gel contained only the Lil3 protein and that Lil3 assembled into the two protein complexes by binding de novo synthesised Chl upon illumination of the etiolated plant.

4. Discussion

In order to identify components involved in binding of the de novo synthesised Chl, we found that none of the photosystem proteins but the light-harvesting like protein Lil3 protein is the primary site to accumulate the main part of de novo synthesized Chl (Fig. 3). The Lil3 protein is a member of the Chl *a*/*b*-binding protein (CAB) family which is characterised by the presence of at least one LHC motif [11]. The LHC motif(s) have been used to group proteins in higher plants as either light-harvesting proteins of the photosystems or as light-harvesting like (Lil) proteins and are the basis for the presumption that all proteins of this group bind Chl *a* and/or Chl *b*.

Up to now, no functional characterization of the Lil3 protein is available. In general, accumulation of Lil proteins were linked with protein translation and photo-oxidative stress [11,12] and proteins were assumed to play a role in protection against reactive oxygen species. Interestingly, Lil3 RNA levels remained unchanged upon transfer of plants to high light whereas for all other members of the Lil family increased mRNA levels were found [11]. Therefore, Lil3 expression and assembly may perform another task in the plant plastid.

According to our finding, assembly of the protein and Chl binding is triggered by illumination of etiolated seedlings. Two functions seem possible. First, Lil3 could act as temporary Chl storage from which single Chl molecules may be delivered to the photosystem proteins on demand. During the first 15 min of deetiolation of higher plants, a 15–35-fold excess of Chl over the amount of Chl-binding proteins from PSI and PSII has been calculated from in vitro assembly studies in isolated etioplasts (unpublished data). Up to now, nothing is known about the delivery of Chlide and geranylgeranylpyrophosphate/phytylpyrophosphate to enzyme CHS and the release of esterified Chl from CHS. Also, no information is available how the Chl released from CHS is delivered to the Chl-binding protein complexes. Since Chl has been discussed to trigger formation of oxygen radicals [13,14], Chl that has not been bound to protein may be prone to rapid degradation. In this respect, a binding of free Chl to specific Chl storage proteins could be required to decrease radical formation and protect the de novo synthesized pigment. Upon illumination, the Lil3 protein has been shown to assemble into two complexes binding Pchl besides Chl (Fig. 2). On the basis of the stability of the Lil3 complexes upon membrane solubilization, the detected molecular mass of the complexes and the fact that only the Lil3 protein, and pigments Car, Chl/Pchl were identified as components of the complexes, we speculate that complexes **2** and **1** constitute a homomultimeric assembly state composed of 7 and 9 protein subunits, respectively. According to the odd number of protein subunits, it appears most likely that Lil3 assembles into two ring-shaped protein complexes upon pigment binding. Pchl had been found recently to bind exclusively to the Cyt b_6f complex in darkness. Hence, the Lil3 protein complexes may act as an intermediary pigment storage compartment upon release of pigments from a protein complex in general. In this direction, ScpD which represents a small CAB-like protein (Scp) in *Synechocystis*, was recently discussed to act as a temporary Chl storage protein in the PS II repair cycle [15]. However, binding of Chl to the ScpD gene product has not been shown directly yet.

Alternatively, Lil3 could act to specifically mediate Chl transfer between CHS and the Chl-binding proteins of PSI and II. Chl transfer between different Chl-binding proteins has been reported [16]; however, no data are available for the transfer of Chl between CHS and PS proteins. For assembly of reaction centre proteins, co-translational binding of Chl to plastid encoded proteins has been suggested [17]; however, it is still unclear whether Chl binding may occur directly after release of Chl from CHS or indirectly via release from a Chl transfer protein. In contrast, the nuclear encoded LHC proteins have been shown to bind Chl in about the correct stoichiometric ratio in an autocatalytic way in vitro and binding was found to be independent from the presence of partner proteins [18].

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